Evans, D.F. (1959), *J. Chem. Soc.*, 2003.
Falk, J.E. (1964), Porphyrins and Metalloporphyrins, New York, N.Y., Elsevier, p 89.
Figgis, B.N. (1966), Introduction to Ligand Fields, New York, N.Y., Interscience, p 248.
George, P., Beetlestone, J., and Griffith, J.S. (1961), *in* Haematin Enzymes, Falk, J.E., Lemberg, R., and Morton, R.K., Ed., London, Pergamon Press, p 105.
Hambright, W.P., Thorpe, A.N., and Alexander, C.C. (1968), *J. Inorg. Nucl. Chem. 30,* 3139.
Handbook of Chemistry and Physics (1968), 48th ed, Weast, R.C., Ed., Cleveland, Ohio, Chemical Rubber Publishing Co., p E.107.
Havemann, R., Haberditzl, W., and Grzegorzewski, P. (1961), *Z. Phys. Chem. 217,* 91.
Hoard, J.L. (1971), *Science 174,* 1295.
Ingram, D.J.E., and Bennett, J.E. (1955), *Discuss. Faraday Soc. 19,* 140.
Kasha, M. (1963), *Radiat. Res. 20.* 55.
Kobayashi, H., Maeda, Y., and Yanagawa, Y. (1970), *Bull. Chem. Soc. Jap. 43,* 2342.
Kobayashi, H., and Yanagawa, Y. (1972), *Bull. Chem. Soc. Jap. 45,* 450.
Lever, A.B.P. (1965), *J. Chem. Soc.*, 1821.
Maricondi, C., Swift, W., and Straub, D.K., (1969), *J. Amer. Chem. Soc. 91,* 5205.
Moss, T.H., Lillienthal, H.R., Moleski, C., Smythe, G.A., McDaniel, M.C., and Caughey, W.S. (1972), *J. Chem. Soc., Chem. Commun.*, 263.
Perutz, M.F. (1970), *Nature (London) 228,* 726.
Seelwood, P.W. (1956), *in* Magnetochemistry, New York, N.Y., Interscience, pp 25, 92.
Smith, D.W., and Williams, R.J.P. (1970), *Struct. Bonding (Berlin) 7,* 1.
Whitten, D.G., Baker, E.W., and Corwin, A.H. (1963), *J. Org. Chem. 28,* 2363.
Zerner, M., Gouterman, M., and Kobayashi, H. (1966), *Theor. Chim. Acta 6,* 363.

Equilibrium and Kinetic Aspects of Subunit Association in Immunoglobulin G†

C. C. Bigelow,‡ B. R. Smith, and K. J. Dorrington*

ABSTRACT: The noncovalent interaction between light chains and dimeric heavy chains from immunoglobulin G myeloma proteins has been followed by difference spectroscopy in 4 mM sodium acetate buffer (pH 5.4). Red-shifted difference spectra were observed consistent with the transfer of aromatic chromophores from a polar to a nonpolar environment upon subunit association. Considerable variation in the shape and magnitude of the difference spectra was noted from one pair of heavy and light chains to another. Tryptophan perturbations were consistently seen but the involvement of tyrosine and phenylalanine transitions was only apparent for some subunit pairs. Equilibrium binding curves were constructed from spectral and calorimetric data which indicated a 1:1 binding between heavy and light chain. The association of monomeric Fd′ with light chain was accompanied by similar changes in molar extinction coefficient, enthalpy, and heat capacity to those seen for reactions involving heavy chain dimer and light chain, thus confirming that the two binding sites for light chain on heavy chain dimer were independent. The time dependence of the difference spectral changes was used to measure the rate of subunit association. The reactions were second order with rate constants between 10^2 and 10^3 l. mol^{-1} sec^{-1} at 25° for several heavy–light chain pairs. The rates showed a marked temperature dependence with Arrhenius activation energies between 16 and 29 kcal mol^{-1}. Fd′, a fragment corresponding to the amino-terminal half of heavy chain, reacted with light chain at about double the rate observed for heavy chain but showed the same temperature dependence. The kinetic data and information on the dissociation of immunoglobulin G at high dilution have been used to estimate a minimum association constant of 10^9 M^{-1} governing the interaction of heavy and light chains. At the concentration of subunits used in this study the half-time for noncovalent association is consistent with the known rate of interchain disulfide bond formation *in vivo*.

The immunoglobulin G (IgG[1]) molecule is composed of two identical H chains (mol wt 53,000) and two identical L chains (mol wt 22,500). The four-chain structure is stabilized by strong noncovalent interactions, between the NH_2-terminal half of an H chain and an L and between the COOH-terminal regions of the two H chains, together with interchain disulfide bonds. During *in vivo* assembly of the molecule noncovalent associations of the subunits must precede interchain disulfide bond formation since sulfhydryl groups must be brought close together before oxidation. The sequence and kinetics of disulfide bond formation have been studied extensively *in vivo* (reviewed by Bevan *et al.*, 1972; Baumal and Scharff, 1973) and, more recently, with an *in vitro* model system (Petersen and Dorrington, 1974). Information on intracellular noncovalent bond formation

† From the Department of Biochemistry and Institute of Immunology, University of Toronto, Toronto, Canada M5S 1A8. *Received June 7, 1974.* Supported by Grant MT4259 from the Medical Research Council of Canada.

‡ Present address: Department of Biochemistry, Memorial University, St. John's, Newfoundland, Canada.

[1] Abbreviations used are: IgG, immunoglobulin G; H, heavy chain; L, light chain; Fd′, a fragment corresponding to approximately the NH_2-terminal half of H chain.

has been difficult to obtain directly and most data have been derived from *in vitro* systems (Cathou and Dorrington, 1974).

Stevenson and Dorrington (1970), using subunits derived from human IgG, found that in low ionic strength buffers at slightly acid pH H chains exist as monodisperse dimers and L chains as a monomer–dimer equilibrium mixture and, further, that mixtures of subunits in such solvents spontaneously reassociate to give a molecule indistinguishable from native IgG. Similar data were subsequently obtained with H and L chains of rabbit IgG (Bjork and Tanford, 1971a–c). Such systems have been used to detect conformational changes (Stevenson and Dorrington, 1970; Bjork and Tanford, 1971c; Dorrington and Smith, 1972) and to evaluate thermodynamic parameters (Smith and Dorrington, 1972; Dorrington and Kortan, 1974) associated with subunit interactions.

In the present study we show that subunit association is accompanied by alterations in the environment of aromatic chromophores as detected by difference spectroscopy. A comparison of the interaction of L chain with either H or Fd′ following both spectral and calorimetric changes yielded identical results indicating that the two L chain binding sites on H chain dimer are independent. This has important consequences for the use of the *in vitro* recombination system as a model for noncovalent assembly *in vivo*. In addition, the time dependence of the spectral changes has been used to determine the kinetics of association.

Materials and Methods

Chemicals. Dithioerythritol and iodoacetamide (recrystallized) were obtained from Sigma Chemical Co. All other reagents were analytical grade. Deionized glass distilled water was used throughout.

Isolation of IgG. Human IgG1 myeloma proteins of both L chain antigenic types (*i.e.*, κ and λ) were isolated from the serum of patients with multiple myeloma by ammonium sulfate precipitation and chromatography on DEAE-cellulose as described previously (Stevenson and Dorrington, 1970). The purity of the final preparations was assessed by cellulose acetate electrophoresis at pH 8.6, immunoelectrophoresis against anti-(whole human serum) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All IgG preparations were stored at −20° in 0.2 M NaCl–0.01 M Tris-HCl (pH 8.0) containing 0.02% sodium azide.

Isolation of Subunits. The reduction and alkylation of IgG, the separation of H and L chains on Sephadex G-100 in 1.0 M propionic acid–25 mM NaCl, and their transfer to 4 mM NaOAc buffer (pH 5.4) were performed exactly as described by Stevenson and Dorrington (1970). Only H chain eluting as a monomer from the Sephadex G-100 was used. The solutions of H and L chains were concentrated to the desired level in an ultrafiltration cell (Amicon Corp.) fitted with an UM-10 membrane.

Preparation of Fd′ Fragment. Fd′ was isolated from $F(ab')_2$, a pepsin-produced fragment of IgG. Digestion of IgG with pepsin at pH 4.5 for 18 hr and isolation of $F(ab')_2$ were performed as described by Palmer *et al.* (1963). $F(ab')_2$ at 10 mg/ml of 0.2 M NaCl–0.01 M Tris-HCl (pH 8.2) was reduced with 10 mM dithioerythritol for 30 min at room temperature and alkylated with 22 mM iodoacetamide for 60 min at 0°. Fd′ and L chain were separated on a column (5.0 × 90.0 cm) of Sephadex G-100 in 1.0 M propionic acid–25 mM NaCl. Under these conditions Fd′ dimerizes and may be separated from the monomeric L chain. Because of incomplete resolution of the two components the leading half of the Fd′ peak and the trailing half of the L chain peak were rechromatographed separately on the same column. These rechromatographed preparations were dialyzed exhaustively against 4 mM NaOAc buffer (pH 5.4) and concentrated to the desired level. The purity of the Fd′ was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel diffusion against anti-L-chain antiserum. The maximum contamination by L chain was never more than 5% in both these tests.

Difference spectra, and the time-dependent changes in them, were measured using a Shimadzu MPS-50L automatic recording spectrophotometer. The temperature in the cuvets was controlled by the circulation of water through cored cuvet holders from an external water bath maintained at the desired temperature ±0.1°.

The protein solutions were placed in rectangular tandem cuvets (Hellma) of total path length 0.878 cm. On the reference side, one compartment of the cuvet held a solution of H chain, the other a solution of L chain. On the experimental side, one compartment held H and L chains mixed at the same concentrations as they occur on the reference side, and the other held buffer.

In the binding curve measurements, the concentration of one of the chains was kept constant throughout, and, in a series of experiments, the concentration of the other was varied so as to give molar ratios of the two components between 0.25 and 1.5. The H–L mixture was allowed to react to equilibrium in the spectrophotometer, and the difference spectrum was recorded. The optical density difference at a convenient wavelength was plotted against the molar ratio of the two components to give a binding curve.

In the kinetic experiments, the solutions, except for the H–L mixture, were placed in the cuvets and the cuvets brought to temperature in the spectrophotometer. At zero time the H–L mixture was prepared and the variation of the absorbance difference (ΔA) at a convenient wavelength (which was usually close to 292 nm) was followed as a function of time, t. The reactions were always completed within 30 min, but in some cases they were too fast to measure by this method, being essentially complete before the first measurement could be recorded. The protein concentrations were usually of the order of 2×10^{-5} M for H chain, and 1×10^{-5} M for L chain.

The absorbancy differences are assumed to be the result of a 1:1 combination of H and L chains, and all our evidence supports this assumption. ΔA_∞, at the end of the reaction, is therefore assumed proportional to the final concentration of HL pairs, which is equal to the concentration of the species, usually L as mentioned above, which was originally present in the smaller molar concentration. On these assumptions, $\Delta A_\infty - \Delta A$ is proportional to [L], the concentration of L at t, and [H] is determined in a straightforward fashion because the difference, [H] − [L], is always the same as the zero-time value $[H]_0 - [L]_0$, if the recombination reaction is a 1:1 process, as our data, and those of others, show it to be.

In this way we have determined ln [H]/[L] over the time course of the reaction studied. This parameter, when plotted against the time, gives a straight line if the reaction is of second order: $1/([H]_0 - [L]_0) \ln [L]_0[H]/[H]_0[L] = kt$. Values of the second-order rate constant k_{12} are determined from the slopes of such curves, and are given in l. mol^{-1} sec^{-1}.

Calorimetric Studies. A batch microcalorimeter (LKB,

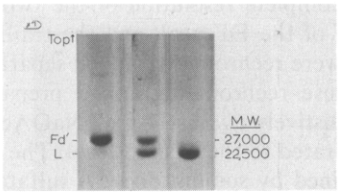

FIGURE 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of Fd′ (left) and L chain (right) from reduced and alkylated F(ab′) (center) separated by gel filtration on Sephadex G-100 in 1.0 M propionic acid–25 mM NaCl. The $F(ab')_2$ was isolated from a pepsin digest of IgG(En).

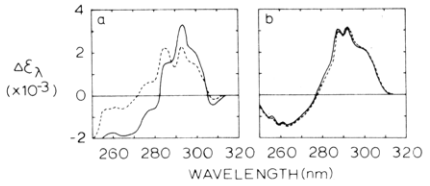

FIGURE 2: Typical examples of difference spectra generated between 250 and 320 nm when H and L chains were recombined in 4 mM NaOAc buffer (pH 5.4) at 25°. (a) H and κ L chains from IgG(Br) (—); H and λ L chains from IgG(Co) (– – –). (b) H and κ L chains from IgG(En) (—). Recombination of Fd′ and L chain from IgG(En) gave a similar difference spectrum (– – –). The change in molar extinction coefficient ($\Delta\epsilon$) was calculated as described in Methods.

Type 10700-2) fitted with 18 karat gold reaction cells was used at 25, 30, and 35°. The differential voltage signal was amplified by a Keithley 150B microvolt ammeter and the amplified signal was recorded on a Recordall 5000 recorder fitted with a ball and disc integrator. The performance of the instrument was checked periodically by measuring the enthalpy of dilution of a standard sucrose solution (Guckert *et al.*, 1939). In all experiments the solvent was 4 mM NaOAc buffer (pH 5.4). The two compartments of the reaction cell were filled with 4.0 ml of H chain (or Fd′) solution and 2.0 ml of L chain solution, respectively. The corresponding compartments of the reference cell were filled with 4.0 and 2.0 ml of buffer. Initial protein concentrations ranged from 4.0 to 4.7 × 10^{-5} M for H chain or Fd′ and 2.5 to 14.0 × 10^{-5} M for L chain. The heats of dilution of Fd′, H, and L chain solutions determined in separate experiments were negligible.

Other Procedures. Protein concentrations were determined spectrophotometrically at 280 nm using the following values for molar extinction coefficients: IgG, 21.0 × 10^4; H, 75.4 × 10^3; Fd′, 35.0 × 10^3; and L, 27.0 × 10^3 (Stevenson and Dorrington, 1970). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn (1969) as described previously (Petersen and Dorrington, 1974); sedimentation velocity data were obtained with a Beckman-Spinco Model E ultracentrifuge, equipped with ultraviolet absorption optics, at 68,000 rpm and 20°. Molecular weights were determined by the method of Yphantis (1964) as described by Dorrington and Mihaesco (1970). Samples of IgG were labeled with carrier-free ^{125}I (New England Nuclear) as described by Smith (1971).

Results

Characterization of Subunits. The physicochemical properties of H and L chains from human IgG in the 4 mM acetate buffer (pH 5.4) have been described in detail by Stevenson and Dorrington (1970). The subunits used in the present study behaved in the same manner.

The method described for the isolation of Fd′ warrants some comments. The successful separation of Fd′ from L depended upon the dimerization of Fd′ in 1.0 M propionic acid. Only three of eight IgG myeloma proteins studied gave $F(ab')_2$ fragments which showed adequate separation of Fd′ and L following reduction and alkylation. The remaining $F(ab')_2$ fragments gave one peak on Sephadex G-100 in 1.0 M propionic corresponding to a mixture of monomeric Fd′ and L chain. We suspect that this inconsistent behavior is related to variable region heterogeneity and we are attempting to correlate it with subgroup specificity.

The preparations of isolated Fd′ showed low-level contamination with L chain on sodium dodecyl sulfate polyacrylamide gel electrophoresis (Figure 1) but gave no reaction at 3 mg/ml with an antiserum to pooled L chain in double diffusion analyses. The molecular weight estimated from the sodium dodecyl sulfate gels was 27,000, assuming a value of 22,500 for L chain. The sedimentation coefficient ($s_{20,w}$) of Fd′ in 4 mM acetate buffer (pH 5.4) was 2.30 (SE ±0.01) at 1.5, 0.75, and 0.25 mg ml^{-1}. At an initial concentration of 0.75 mg ml^{-1} a molecular weight of 28,000 was obtained by high-speed sedimentation equilibrium method (Yphantis, 1964). Therefore, at the concentrations used in the recombination reactions, Fd′ was predominantly monomeric.

Difference Spectra. When the ultraviolet absorption of recombined H and L chains was compared to identical concentrations of separated subunits, between 240 and 320 nm, difference spectra were observed as shown in Figure 2. These examples were chosen to illustrate various features of the spectra. All spectra are characterized by positive peaks above 270 nm due to a reduction in the energy of aromatic tranitions (*i.e.*, "red-shifted" spectra). It is apparent, however, that the spectra differ in magnitude and overall shape from one protein to another. All the spectra exhibit a strong peak near 292 nm corresponding to a difference in molar extinction coefficient ($\Delta\epsilon$) between 2000 and 3000. This feature can be unambiguously assigned to a perturbation of the ^1L_b band of the indole chromophore in tryptophan (Donovan, 1969). The spectra observed for IgG(En) and IgG(Co) showed evidence for perturbation of tyrosine as judged by the magnitude of the band at 288 nm. Involvement of phenylalanine is suggested by the characteristic series of weak bands between 255 and 265 nm in IgG(En). The subunits of protein Br give a tryptophan spectrum with little evidence for the involvement of other chromophores. It should be noted that although the recombination reaction appears to be $H_2 + 2L \rightleftharpoons H_2L_2$ all the data presented have been calculated for the half-reaction, $H + L \rightleftharpoons HL$, assuming independent sites for binding L chain to H chain dimer (see Discussion).

Subunit binding curves were constructed from the difference spectra as shown in Figure 3. The value of $\Delta\epsilon$ increased as a linear function of L chain concentration until equimolar amounts of H and L were present after which there was no further change. To calulate $\Delta\epsilon$ we used the concentration of HL which was assumed to be equal to [L] up to [H] = [L] and equal to [H] at higher [L]. Exactly similar curves were obtained when the final L chain concentration was held constant and H varied. Two general conclusions may be drawn from these binding data. Firstly, the linearity and sharp inflection at the equimolar concentrations point to the high affinity of the 1:1 noncovalent interaction between H and L. Secondly, essentially all the H

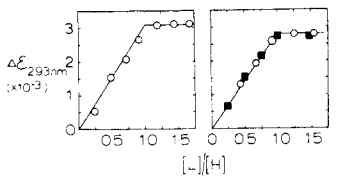

FIGURE 3: Equilibrium binding curves obtained from the difference spectra generated at different molar ratios of H and L chains at 25°. Final [H] was held constant (2×10^{-5} M) and the final [L] was varied to give the molar ratios indicated. Left: H and L from IgG(Br). Right: H and L (○); Fd′ and L(■) from IgG (En). Values of $\Delta\epsilon_{293nm}$ were calculated from the concentration of the subunit present in the lower concentration as described in Methods.

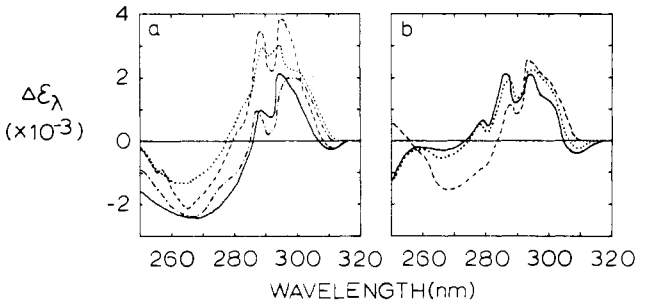

FIGURE 4: Comparison of difference spectra generated in autologous and heterologous recombination reactions. (a) H(En) recombined with L(En) (····) and three other κ L chains: L(Br) (—); L(G1) (- · -), and L(Te) (- - -). (b) H(Co) recombined with L(Co) (—) and two other λ L chains: L(Pi) (- - -) and L(No) (····).

chain is capable of binding L thus confirming the efficacy of the acetate buffer system used in inhibiting the aggregation of H chain.

Data on the association of Fd′ and L chain are also shown in Figures 2 and 3. The magnitude and shape of the difference spectrum generated when Fd′ and L interacted were identical with that for H and L association (Figure 2) as was the binding curve (Figure 3).

The spectral changes presented above accompanied autologous[2] recombination reactions. A limited number of heterologous reactions was also studied (Figure 4). In one series, H(En) chains were recombined separately with κ L chains from three other IgG proteins. The spectra observed are shown in Figure 4a. Reassociation of H(En) with L(Te) have difference spectra similar to those obtained in the autologous reaction with involvement of both tryptophan and tyrosine. Association of either L(G1) or L(Br) with H(En) produced similar spectra but these were markedly different from the autologous system being dominated by tryptophan perturbation. In a second series, H(Co) chains were reacted separately with two other λ L chains. As shown in Figure 4b L(Pi) gave a difference spectrum similar to the autologous reaction whereas L(No) yielded spectral changes which showed little evidence of tyrosine involvement.

Microcalorimetry. The enthalpy changes ($\Delta H°$) accompanying the interaction of several H and L chains have been reported earlier (Dorrington and Kortan, 1974). In the present study we have compared the heats evolved in reactions between either intact H chain dimer or Fd′ fragment and L chain. The binding curves obtained when progressively higher concentrations of L chain were mixed with either

[2] "Autologous" refers to reactions between isolated H and L chains derived from the same myeloma protein and "heterologous" described reactions between subunits derived from different myeloma proteins.

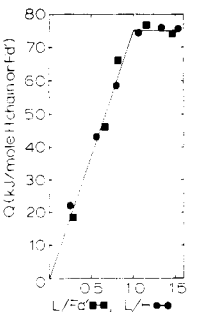

FIGURE 5: Calorimetric binding curves for H and L (●) and Fd′ and L (■) from IgG(En) at 25° in 4 mM NaOAc buffer (pH 5.4). The final concentration of either H or Fd′ was kept constant (approximately 3.0×10^{-5} M) and the concentration of L was varied to give the molar ratios indicated. Each value of the heat evolved (Q) is the mean of at least three determinations.

TABLE I: Enthalpy ($\Delta H°$) of Association between EnL Chain and Either EnH Chain or EnFd′ at Different Temperatures.

Reaction	$-\Delta H°$ (kJ mol^{-1})[a] at 25°	30°	35°	$-\Delta C_p$ (kJ deg^{-1} mol^{-1})
EnH + EnL	75.0 ± 6.0	124.0 ± 7.0	177.0 ± 10.0	10.2
EnFd′ + EnL	73.0 ± 3.0	122.0 ± 5.0	171.0 ± 9.0	9.8

[a] Calculated for each mole of either HL or Fd′L formed. Mean of at least three determinations ± standard error.

Fd′ or H chain are shown in Figure 5. It is clear that Fd′ and H chain dimer interact with L chain in an identical way. The temperature dependence of the enthalpy of binding and the associated values for the change in heat capacity (ΔC_p) are also the same for reactions involving either Fd′ or H chain (Table I).

Other Equilibrium Studies. It was apparent from the binding curves obtained from the spectral and calorimetric data that neither of these methods could be used to measure association constants governing the interaction of H and L chains. We attempted to approach this problem by studying the dissociation of noncovalently assembled IgG. Such studies could be performed at low protein concentrations by using high specific activity [^{125}I]IgG. A typical experiment proceeded as follows. Intact [^{125}I]IgG at an initial concentration of approximately 5×10^{-10} M was passed over a column of Sephadex G-100 (49 × 2.5 cm) in 0.15 M NaCl, 10 mM Tris-HCl (pH 8.0) containing 2 mg/ml of bovine serum albumin as carrier. This eluted from the column as a single symmetrical peak (Figure 6). Another aliquot of the same [^{125}I]IgG was reduced and alkylated, diluted to about 5×10^{-10} M, and applied to the Sephadex G-100 as before. Although varying lengths of time (1–48 hr) were allowed to elapse between dilution and gel filtration, in all cases the radioactivity appeared at an elution position identical with the intact IgG (Figure 6). No radioactivity appeared at an elution position corresponding to L chain. Since counts at this position was our criterion for dissociation, a sample of the reduced and alkylated [^{125}I]IgG was chromatographed on Sephadex G-100 in 1.0 M propionic acid. Radioactivity was present on both the H and L chains (Figure 6, inset).

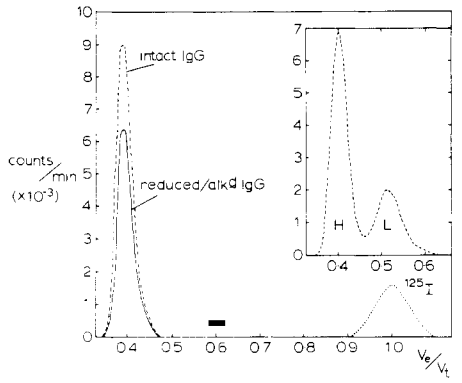

FIGURE 6: Gel filtration of intact [^{125}I]IgG (- - -) and mildly reduced and alkylated [^{125}I]IgG (—) on Sephadex G-100 in 0.15 M NaCl–10 mM Tris-HCl buffer (pH 8.0) containing 2 mg ml^{-1} of bovine serum albumin. The initial protein concentration was approximately 5×10^{-10} M. The total volume (V_t) of the column was marked with free ^{125}I. The solid bar indicates the elution position of free L chain on this column. Inset: Gel filtration of a sample of reduced and alkylated [^{125}I]IgG on Sephadex G-100 in 1.0 M propionic acid–25 mM NaCl.

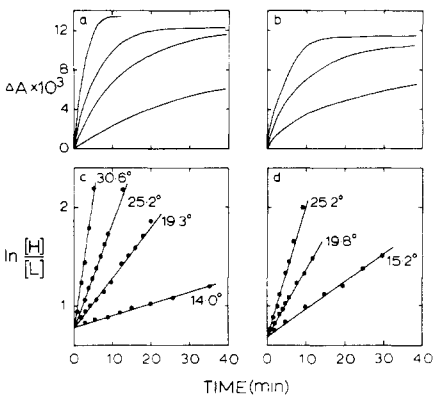

FIGURE 7: Kinetics of association for H and L (a and c) and Fd′ and L (b and d) from IgG(En) at various temperatures. The change in absorbance (ΔA) at 292 nm is plotted as a function of time (a,b). At each temperature the data are fitted to a second-order rate expression (see Methods) as shown in c and d.

Kinetic Studies. An approach to evaluating the kinetics of subunit association was suggested initially by the observation that the maximum difference spectral changes were not attained until several minutes after the H and L chains were mixed. This is illustrated in Figure 7a where the magnitude of the difference peak at 292 nm is shown as a function of time at four temperatures. The rate is markedly temperature dependent over the range studied (14–35°). Comparable data for Fd′ is also shown in Figure 7b. When these data were analyzed using a second-order rate expression (see Methods) linear plots were obtained at all temperatures (Figure 7c,d). Some representative forward rate constants (k_{12}) for the reaction

$$H + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} HL$$

are given in Table II.

Arrhenius plots describing the temperature dependence of the rate of interaction of EnL with either EnH or EnFd′ are seen in Figure 8. Fd′ and H show essentially the same temperature dependence, with an activation energy of 26 kcal mol^{-1}, but Fd′ reacts with L at approximately double the rate of H chain. The effect of temperature on the half-time ($t_{1/2}$) of the reaction between H(En) and L(En) is seen in Figure 9; ln $t_{1/2}$ is a linear function of temperature. At 15° $t_{1/2}$ is approximately 16 min while at 35° it has decreased to 1.6 min.

Discussion

The recombination of H and L chains from human IgG in 4 mM acetate buffer at pH 5.4 appears to involve reactions 1–4 (Stevenson and Dorrington, 1970).

$$L_2 \rightleftharpoons 2L \quad (1)$$

$$H_2L + L \rightleftharpoons H_2L \quad (2)$$

$$H_2 + L \rightleftharpoons H_2L_2^* \quad (3)$$

$$H_2L_2^* \rightleftharpoons H_2L_2(\equiv IgG) \quad (4)$$

Briefly, the evidence is as follows. At the concentrations usually employed ($2.0–8.0 \times 10^{-5}$ M) H chain is a noncovalent dimer and L chain a monomer–dimer equilibrium mixture by a number of physicochemical criteria. The involvement of monomeric L chain as the interacting species was suggested by the observation that disulfide-linked L dimers would not recombine with H chain. In addition the equilibrium constant governing reaction 1 is approximately 10^4 M^{-1} (Green 1973; B. R. Smith and K. J. Dorrington, unpublished data) so that at the usual concentrations L chain would be present predominantly as the monomer. H chain appears to be a dimer at concentrations as low as 10^{-10} M due to strong interactions within the Fc region (B. R. Smith and K. J. Dorrington, unpublished data) and further stabilization of the dimer with interchain disulfide

TABLE II: Some Representative Forward Rate Constants (k_{12}) for the Interaction of H and L Chains in 4 mM NaOAc Buffer (pH 5.4) at Several Temperatures.

Reaction	k_{12}[b] (l. mol^{-1} sec^{-1}) at 15°	20°	25°	30°	35°	E_a[a] (kcal mol^{-1})
EnH + EnL	30	70	140	290	n.d.	26
EnFd′ + EnL	60	120	230	520	n.d.	26
EnH + G1L	50	90	155	220	n.d.	16
EnH + BrL	30	70	130	200	300	18
BrH + BrL	60	160	320	n.d.	n.d.	29
CoH + CoL	>1000	n.d.	>1000	n.d.	n.d.	
CoH + PiL	>1000	n.d.	>1000	n.d.	n.d.	

[a] Arrhenius activation energy calculated from the slope of the correlation between ln k_{12} and $1/T$ (°K). [b] n.d. = not determined.

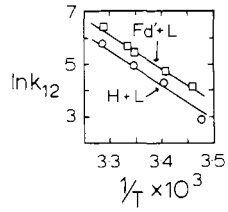

FIGURE 8: The temperature dependence of the second-order forward rate constant (k_{12}) for the association of H + L or Fd' + L from IgG(En). T is in °K.

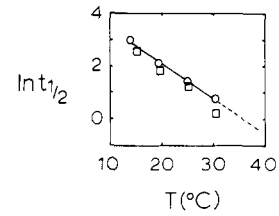

FIGURE 9: The half-time ($t_{1/2}$) for the association of H and L (○) or Fd' and L (□) as a function of temperature.

bonds has no measurable effect on recombination efficiency (Stevenson and Dorrington, 1970). Reaction 4 in the above scheme represents an isomerization to account for the conformational changes accompanying subunit interaction (Dorrington *et al.*, 1967; Stevenson and Dorrington, 1970; Bjork and Tanford, 1971c; Dorrington and Smith, 1972).

In reactions involving Fd' and L chain the following scheme (eq 5-8) may be proposed. Human Fd' is weakly

$$L_2 \rightleftharpoons 2L \quad (5)$$

$$Fd'_2 \rightleftharpoons 2Fd' \quad (6)$$

$$Fd' + L \rightleftharpoons Fd'L^* \quad (7)$$

$$Fd'L^* \rightleftharpoons Fd'L(\equiv Fab) \quad (8)$$

self-associating with a dimerization constant near 10^4 M^{-1}. Rabbit Fd', although obtained from a heterogeneous population of IgG molecules, appears to behave in the same fashion (Huston *et al.*, 1972). Thus, at the concentrations employed in the current study the reactions studied involve the monomeric forms of Fd' and L chain.

It was important to establish, particularly in the interpretation of the kinetic data, that the two sites for binding L chain on the H chain dimer were independent. The form of the binding curves obtained from both the difference spectral and calorimetic data strongly supported this assumption. The most convincing observation was that these curves were identical in reactions involving either intact H chain dimer or monomeric Fd' fragment as judged by changes in molar extinction coefficient, enthalpy, and heat capacity.

Interpretation of the spectral changes in structural terms is hampered by our limited knowledge regarding the fine structure of IgG. The simplest analysis of the red-shifted difference spectra would suggest that aromatic chromophores, which are exposed to bulk solvent in the free subunits, become buried in regions of low polarity upon H-L interaction. On the basis of model compound studies, the burial of a fully exposed tryptophanyl side chain would give a value of about 1600 for $\Delta\epsilon_{292nm}$ (Donovan, 1969). The values of $\Delta\epsilon_{292nm}$ observed in this study could correspond to the burial of up to two tryptophans for each H-L or Fd'-L pair formed. Is this reasonable from what we know of IgG structure? The high resolution X-ray crystallographic data on Fab' (New) (Poljak *et al.*, 1973) indicates that Trp-49 and Trp-107 on Fd' are present at the interface between the two subunits. This observation supports a simple interpretation of the spectral data. However, the results of the heterologous recombinations suggest a more complex situation. For example, H(En) chain gave evidence for the involvement of two tryptophans ($\Delta\epsilon_{292}$ = 3000) when reacted with L(En) or L(Tc) but only one tryptophan ($\Delta\epsilon_{292nm}$ = 2000) with L(G1) and L(Br), as well as differences in the extent to which tyrosine might be involved. While an extensive discussion of these observations does not seem appropriate considering the limitations of our knowledge, we would suggest that some of the spectral changes may reflect alterations in the environment of tryptophanyl and other side chains due to conformational changes within one or both of the subunits. The existence of such changes have been inferred from both optical rotatory dispersion and circular dichroism studies on the free and associated subunits (see Cathou and Dorrington, 1974).

The difference spectra also provide clear evidence for charge perturbation of indole transitions. Ananthanarayanan and Bigelow (1969a,b) have shown that absorbancy differences frequently observed in protein difference spectra near 300 nm are due to charge effects on tryptophan and suggested that positive differences arise if tryptophan separates from a positive charge or approaches a negative one. All spectra observed in the present study exhibited positive differences near 300 nm although negative differences were noted in some spectra at longer wavelength. Further analysis of the protein models derived from X-ray investigations for close association between indole and carboxylate groups may be extremely helpful in determining the structural basis for the spectral changes.

From considerations of reactions 1 to 4 given above, the difference spectra observed under equilibrium conditions could have resulted from either the interaction of the subunits or a conformational change occurring subsequent to interaction. The finding that the time course of the spectral change followed second-order kinetics strongly suggested that H-L interaction *per se* is the rate-limiting event. A rate-limiting conformational change would have presumably imposed first-order kinetics on the overall reaction. Similar arguments can be applied to the association of Fd' and L chain (*i.e.*, reactions 5-8). We have no way of determining from the spectral data whether the conformational changes referred to above occur concomitantly with association or subsequently.

One of the surprising results of this study was the low rate at which H and L chains interacted. At 25° the values for k_{12} varied from less than 10^2 to more than 10^3 l. mol^{-1} sec^{-1}. The latter figure represents the upper limit for the method used to follow kinetics.[3] It is tempting to attribute these differences to interactions between the variable regions of the subunits. A correlation of variable region subgroup and forward rate constant would be extremely interesting.

Fd' reacted with L chain at approximately double the

[3] Subunit association is also accompanied by a small (<15%) quenching of intrinsic tryptophan fluorescence. Some preliminary attempts have been made to estimate k_{12} by stopped-flow measurements for one IgG and a value near 10^4 l. mol^{-1} sec^{-1} was obtained. The range of rate constants therefore may be at least two orders of magnitude.

rate observed for H chain. This may reflect the greater probability of nonproductive collisions between L and H chain dimer than between L and Fd′. The slower rotational relaxation time of H dimer may also be a contributing factor. Orientation effects are obviously important in both systems since the observed rate constants are far below those of diffusion-controlled processes (*i.e.*, 10^8–10^9 l. mol^{-1} sec^{-1}).

The slow association rates may impose limitations on the interpretation of the experiments designed to measure dissociation of noncovalently assembled IgG. Our failure to detect dissociation at concentrations of IgG near 10^{-10} M might suggest that the apparent association constant (K_A) governing H–L interaction was greater than 10^{10} M^{-1}. Taking such a value and $k_{12} = 10^2$ l. mol^{-1} sec^{-1} we can calculate a half-time for dissociation of about 2.0×10^4 hr at 25°. Thus only about 0.15% dissociation would have been anticipated even after the longest period (48 hr) which elapsed between reduction and alkylation and gel filtration. Some estimate of the minimum value for K_A can be obtained from such calculations. For $k_{12} = 10^2$, K_A must be at least 10^9 M^{-1} for no measurable dissociation to have been detected after 48 hr at 10^{-10} M. In this connection it is interesting to recall that the isolated variable and constant regions of L chain each interact with H chain with an apparent association constant of about 10^4 M^{-1} (Smith and Dorrington, 1972). On this basis intact L chain would interact with H with a $K_A = 10^8$ M^{-1}.

One of the primary reasons for studying the association of subunits *in vitro* has been to generate models for the *in vivo* assembly of IgG. Only limited information has been gained regarding the noncovalent interaction of subunits from *in vivo* studies. Noncovalent association of L chain with nascent H chain on heavy polyribosomes has been demonstrated (Shapiro *et al.*, 1966; Schubert, 1968; Askonas *et al.*, 1969) but it is not known whether or not this precedes dimerization of the H chains. It was this uncertainty which prompted our comparative studies on Fd′ and H chain interactions with L chain. As we have already pointed out our data suggest that the interaction of L chain with Fd′ is largely insensitive to whether the Fd′ region is isolated or forms a part of H chain dimer. Accepting the validity of this conclusion, are the *in vitro* rates consistent with what we know about the kinetics of IgG assembly *in vivo*? From a variety of studies, mainly with mouse tumor cell systems, the average half-time for covalent assembly of IgG is near 10 min at 37° (Baumal and Scharff, 1973) and similar values have been obtained *in vitro* (Petersen and Dorrington, 1974). The half-time for noncovalent assembly must be no greater than this value. In fact, the extrapolated half-time for H–L interaction at 37° was 0.75 min *at the concentration of subunits used in this study* (*i.e.*, $\sim 10^{-5}$ M). Our degree of confidence in the *in vitro* model would be enhanced if the local concentration of subunits in the cisternae of the endoplasmic reticulum were known. Unfortunately they are not. A comparison of noncovalent assembly *in vivo* and *in vitro* is further complicated by the difference in pH between the two systems. While no significant difference in forward rate constant could be detected for one H–L pair between pH 4.5 and 6.5 we cannot be sure that this insensitivity extends into the physiological pH range. Above pH 6.5 the aggregation of the H chains complicates the spectral measurements.

Acknowledgments

We thank Dr. W. Pruzanski, Wellesley Hospital, Toronto, for generous supplies of serum. The technical assistance of Miss Cathy Kortan was invaluable.

References

Ananthanarayanan, S., and Bigelow, C. C. (1969a), *Biochemistry 8,* 3717.

Ananthanarayanan, S., and Bigelow, C. C. (1969b), *Biochemistry 8,* 3723.

Askonas, B. A., Williamson, A. R., and Awdeh, Z. L. (1969), *Gamma Globulins: Struct. Biosyn., Fed. Eur. Biochem. Soc., Meet., 5th, 1968,* 105.

Baumal, R., and Scharff, M. D. (1973), *Transplant. Rev. 14,* 163.

Bevan, M. J., Parkhouse, R. M. E., Williamson, A. R., and Askonas, B. A. (1972), *Progr. Biophys. Mol. Biol. 25,* 131.

Bjork, I., and Tanford, C. (1971a), *Biochemistry 10,* 1271.

Bjork, I., and Tanford, C (1971b), *Biochemistry 10,* 1280.

Bjork, I., and Tanford, C. (1971c), *Biochemistry 10,* 1289.

Cathou, R. E., and Dorrington, K. J. (1974), *in* Biological Macromolecules, Fasman, G. D., and Timasheff, S. N., Ed., New York, N.Y., Marcel Dekker (in press).

Donovan, J. W. (1969), *in* Physical Principles and Techniques in Protein Chemistry, Part A, Leach, S. J., Ed., New York, N.Y., Academic Press, p 101.

Dorrington, K. J., and Kortan, C. (1974), *Biochem. Biophys. Res. Commun. 56,* 529.

Dorrington, K. J., and Mihaesco, C. (1970), *Immunochemistry 7,* 651.

Dorrington, K. J., and Smith, B. R. (1972), *Biochim. Biophys. Acta 263,* 70.

Dorrington, K. J., Zarlengo, M. H., and Tanford, C. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 996.

Green, R. W. (1973), *Biochemistry 12,* 3225.

Guckert, F. T., Pichard, H. B., and Plank, R. W. (1939), *J. Amer. Chem. Soc. 61,* 459.

Huston, J. S., Bjork, I., and Tanford, C. (1972), *Biochemistry 11,* 4256.

Palmer, J. L., Nisonoff, A., and Van Holde, K. E. (1963), *Proc. Nat. Acad. Sci. U. S. 50,* 314.

Petersen, J. G. L., and Dorrington, K. J. (1974), *J. Biol. Chem.* (in press).

Poljak, R. J., Amzel, L. M., Avey, H. P., Chem, B. L., Phizackerley, R. P., and Saul, F. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 3305.

Schubert, D. (1968), *Proc. Nat. Acad. Sci. U. S. 60,* 683.

Shapiro, A. L., Scharff, M. D., Maizel, J. V., and Uhr, J. W. (1966), *Proc. Nat. Acad. Sci. U. S. 56,* 216.

Smith, B. R. (1971), *Biochim. Biophys. Acta 229,* 649.

Smith, B. R., and Dorrington, K. J. (1972), *Biochem. Biophys. Res. Commun. 46,* 1061.

Stevenson, G. T., and Dorrington, K. J. (1970), *Biochem. J. 118,* 703.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

Yphantis, D. A. (1964), *Biochemistry, 3,* 297.